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Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Body.....	4
Key Research Accomplishments.....	6
Reportable Outcomes.....	7
Conclusions.....	7
References.....	7
Appendix 1.....	9

4. BODY

The overall goal of this research is to understand the importance of the interaction between Smad3 and the estrogen receptor (ER) as it pertains to human breast tumorigenesis and breast cancer progression. The ER family consists of the classical ER, ER α , and the recently described ER β . Although the definitive roles of ER α in the development and progression of breast cancer have been partially elucidated, the role of ER β remains unknown. However, ER β and ER β variant mRNA and proteins have been identified in breast cancer cell lines as well as in both normal and neoplastic human breast tissues^{1, 2, 3, 4, 5, 6}. In normal breast tissue, expression of ER β is more frequently and likely higher compared to ER α while during breast tumorigenesis, the relative expression of ER β :ER α decreases⁵. Therefore, ER β and its variants may play an important role in normal breast tissues that is altered in breast tumorigenesis and the identification of factors that interact with ER may help to identify a role for ER β . Preliminary experiments from our laboratory using the yeast two-hybrid system suggested that an interaction between ER α , ER β_1 , and ER β_2 with Smad3 may exist⁷. Smad3 is a downstream signaling protein of the transforming growth factor β (TGF β) signaling cascade that has previously been shown to interact with the androgen (AR)^{8, 9}, glucocorticoid (GR)¹⁰ and vitamin D (VDR)¹¹ receptors. AR, GR and VDR all belong to the steroid nuclear receptor superfamily to which the ER also belongs, further suggesting that a cross-talk between ER and TGF β may occur. Therefore, we proposed to test the hypothesis that ER β and/or its variants directly interact with the TGF β signal transduction pathway and is part of the mechanism through which ER β and/or its variants negatively modulate the ER α proliferative signal transduction pathway.

Our first specific aim was to define the specificity of the interaction of Smad3 with the ER family, both *in vitro* and *in vivo* using co-immunoprecipitations (co-IPs). We have cloned the full length open-reading frame of ER α , ER β_1 , ER β_2 , and ER β_5 in frame with the N-terminal 6 x histidine and xpress tagged expression vector pcDNA4 (Invitrogen) and confirmed the constructs to be in-frame. Earlier last year we were kindly given Smad2, 3 and 4 expression plasmids from Dr. Attisano (University of Toronto) and these have also been confirmed by sequencing. To determine whether an ER/Smad3 interaction occurs *in vitro*, we *in vitro* transcribed/translated S³⁵ radiolabelled ER and Smad3 using the TnT coupled reticulocyte lysate system (Promega). Proteins were mixed on ice in the presence or absence of 10nM estradiol and immunoprecipitated (IP) with either ER α , His (recognizes the histidine residues on the tagged ER) or β -galactosidase (negative control). IPs with a Smad3 specific antibody were not performed *in vitro* as this antibody was raised in rabbits and our reticulocyte lysate in which we produce our proteins is also rabbit. IP products were run on a 10% SDS-Page gel and visualized by autoradiography. Our results suggest that an interaction between ER α and ER β_1 with Smad3 may exist, although the interaction appears to be weak. No interaction between the ER β variants, ER β_2 and ER β_5 , with Smad3 occurred *in vitro* under these conditions. Several attempts were also made to determine the interaction *in vivo* in Cos1 cells. Cells were transfected with ER and Smad3 in the presence or absence of 10nM

estradiol and subsequently the cell lysates were IP for either ER or Smad3. Results from these experiments suggested that an interaction between ER α and ER β_1 with Smad3 did not occur *in vivo* in Cos1 cells or that the interaction was too weak to be detected using the IP methodology. Recently however, Matsuda et al.¹² described the physical interaction between Smad3 and ER. Through a series of co-IPs, these authors suggest that Smad3 interacts with ER α and ER β_1 in 293T and MCF-7 cells and that this interaction is dependent on activation of both the estrogen and TGF β signaling cascades. Further studies have shown that ER α and ER β_1 interact with Smads 1, 2, 3 and 4 upon stimulation of the ER and TGF β pathways^{12, 13}. Therefore, cell type specific factors may influence the interaction of ERs and Smad3.

In our original research proposal, we next wanted to examine the structural/functional regions of ER and Smad3 that are required for binding. Although Matsuda et al. do not describe the region of the ER that specifically binds Smad3, they do demonstrate that the DNA binding domain of ER is involved in its interaction with Smad1¹³. In addition, these authors have shown that the MH2 domain of Smad3 binds ER¹².

We next wanted to determine whether the interaction between Smad3 and ER affects ER transcriptional activity. We have obtained a vitellogenin estrogen responsive (ERE) luciferase reporter plasmid from the laboratory of Dr. Webb (University of California). To ensure that the plasmid was indeed ER responsive, we transiently transfected Cos1 cells with the ERE, ER α and the β -galactosidase reference gene pCH110, in the presence or absence of 10nM estradiol. Indeed, results indicate that upon estradiol stimulation, there was a 7 fold increase in luciferase activity. When increasing amounts of Smad3 were co-transfected into Cos1 cells with the ERE, ER α and pCH110 in the presence or absence of 10nM estradiol, no significant difference in luciferase activity between samples treated with Smad3 and ER α compared to those treated with ER α alone was observed. Similar results were also obtained when ER β_1 was transfected rather than ER α . As data from our laboratory suggests that the ER β variants do not have transcriptional activity of their own¹⁴, ERE-reporter genes have not been used in conjunction with the variants. Our observation that Smad3 does not affect ER transcription on the vitellogenin ERE is in agreement with several other laboratories^{3, 4}. However, Matsuda et al.¹² suggest that Smad3 increases ER transcriptional activity. Although the discrepancy between these results is unknown, it may be due to the different cellular environments in which the experiments were performed or to differences in reporter genes. We are currently in the process of confirming our results in the T5 human breast cancer cell line which contains endogenous ER α and are TGF β responsive. However, these cells have proven to be extremely difficult to transiently transfect and we are currently testing several transfection methods to overcome this hurdle. The vitellogenin ERE-reporter plasmid described above represents a classical ERE, in which ER directly binds to the DNA. However, ER has also been shown to regulate target gene transcription in a non-classical manner, in which the ER interacts with other proteins (i.e. c-Jun) that then bind to DNA¹⁵. We currently have available to

us TGF β ₃ and Ap-1 regulated promoters that represent non-classical EREs and we are in the process of testing the effect of Smad3 and ER on the activity of these plasmids.

Although Smad3 does not affect ER transcription in Cos1 cells, the question still remains whether ER could affect Smad3 transcriptional activity. Recently, we obtained the Smad3 responsive p3TP-Lux luciferase reporter plasmid from Dr. Massague (Rockefeller University) which contains the TGF β responsive element of the plasminogen activator inhibitor-1 (PAI-1) downstream of three TPA-responsive elements of the human collagenase gene¹⁶. This plasmid has been well characterized as a TGF β responsive promoter and overexpression of Smad3 by transient transfection increases its activity^{16, 17, 18, 19}. We have transfected this plasmid into Cos1 cells along with Smad3, ER and pCH110 as a transfection efficiency control in the presence or absence of 10nM estradiol. Results indicate that ER α (p<0.001) and ER β ₁ (p<0.05) inhibit p3TP-Lux transcription in the presence of estradiol. To assess the specificity of estrogen effects on Smad3 transcriptional activity, we utilized the anti-estrogens tamoxifen and ICI 182,780. The inhibitory effect of ER α and ER β ₁ in the presence of estradiol on p3TP-lux was reversed by both 4OH-tamoxifen (100nM) and ICI 182,780 (100nM) suggesting that the effect of ER on Smad3 transcription is ligand dependent. ER β ₂ and ER β ₅ did not affect Smad3 transcriptional activity. However, upon western blot analysis of our transfected cells, it appears as though ER β ₂ and ER β ₅ are expressed at a much lower level compared to ER β ₁ and ER α which may account for the differences. Although this is unlikely since the variant receptors are not ligand activated, we are currently attempting to increase the protein expression level of these variants in our Cos1 cells to determine whether this higher level of expression affects Smad3 transcriptional activity on p3TP-Lux.

4. KEY RESEARCH ACCOMPLISHMENTS

- ♦ Cloning of ER α , ER β ₁, ER β ₂, ER β ₅ and Smad3 into appropriate vectors.
- ♦ Co-immunoprecipitation assays *in vitro* and in Cos1 cells completed.
- ♦ Optimization of ERE-luc assays in Cos1 cells.
- ♦ Smad3 does not affect ER transcriptional activity on a vitellogenin ERE regulated promoter in Cos1 cells.
- ♦ Optimization and validation of the Smad3 reporter gene, p3TP-Lux.
- ♦ ER α and ER β ₁ inhibit Smad3 transcription on p3TP-Lux in a ligand-dependent manner and this effect is prevented in the presence of the anti-estrogens 4OH-tamoxifen and ICI 182,780.
- ♦ ER β ₂ and ER β ₅ do not appear to affect Smad3 transcription on the p3TP-lux reporter.

5. REPORTABLE OUTCOMES

Cherlet T, Murphy LC (2002). Cross-talk between the transforming growth factor beta (TGFbeta) and estrogen receptor (ER) signaling pathways in human breast cancer. Abstract presented at Research Day 2002, June 4-6, 2002, Winnipeg, Manitoba. **Appendix 1.**

6. CONCLUSIONS

Our preliminary experiments together with our current results suggest that a cross-talk between the ER and TGFβ signalling pathways exists. Our preliminary data suggested that an interaction between Smad3 and ER exists in the yeast two-hybrid system. Our current data suggests that a weak physical interaction between Smad3 and ERα or ERβ₁ exists *in vitro*, although we have not been able to detect this *in vivo* in Cos1 cells. Furthermore, increased Smad3 expression does not effect ER transcriptional activity as measured through activation of an ERE luciferase reporter gene. However, ER is able to repress Smad3 transcriptional activity on the Smad reporter gene p3TP-lux in a ligand dependent manner. The mechanisms by which this occurs are currently being investigated. Overall the data support the hypothesis that ER interacts with the TGFβ signalling pathway.

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Appendix 1

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CROSS-TALK BETWEEN THE TGF- β AND ER SIGNALING PATHWAYS.

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The ER family consists of ER α and ER β . In normal breast tissues, expression of ER β is high while ER α levels are low. During breast tumorigenesis, however, ER β expression decreases while ER α increases. Therefore, ER β may play an important role in normal breast tissues that is altered in breast tumorigenesis. Results from a yeast two-hybrid screen suggest that ER β interacts with Smad3, a signalling protein of the TGF β cascade. Although TGF β normally negatively regulates cellular proliferation, many breast cancers are resistant to TGF β . As Smad3 interacts with other members of the steroid nuclear superfamily, cross-talk between the TGF β and ER pathways may exist. We hypothesize that ER β interacts with the TGF β pathway and that this interaction modulates TGF β signaling. Initially, we examined interactions between ER and Smad3 *in vitro*. ER and Smad3 were radiolabelled using a coupled transcription/translation system and immunoprecipitated. When low levels of ER α were present, an interaction was observed while at high ER α levels, the interaction was abolished. An

interaction between ER β and Smad3 was also observed. Secondly, we examined whether cross-talk between Smad3 and ER alters Smad3 or ER activity. Cos1 transient transfections with an ERE-Luc suggest that Smad3 does not affect ER α nor ER β transcription. However, ER α and ER β inhibited Smad3 (p3TP-Lux) transcription in a ligand-dependent fashion. As ER expression and TGF β activation alter during breast tumorigenesis, cross-talk between these pathways may have a role in breast tumorigenesis.